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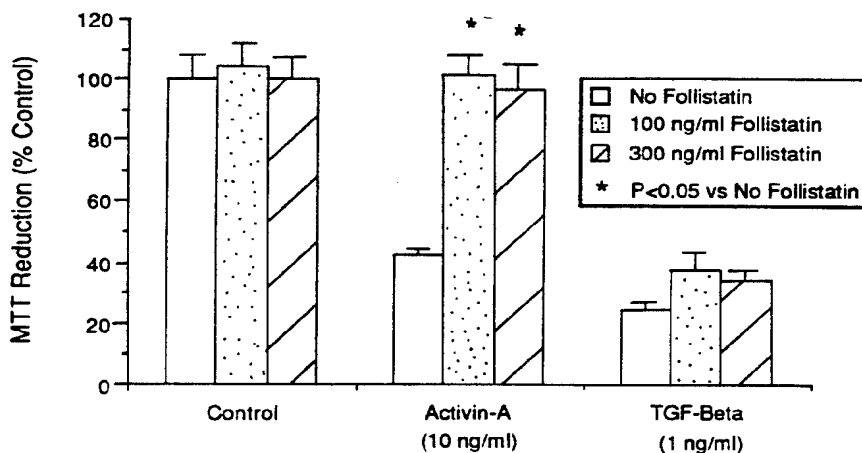
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(54) Title: METHOD FOR PREVENTING OR TREATING LIVER DISEASE



(57) Abstract

The invention involves a method for preventing hepatic injury or liver cell death associated with certain liver diseases in a mammal comprising administering to the mammal an effective amount of an antagonist to activin, optionally in combination with other antagonists to hepatocytotoxic proteins such as TGF- $\beta$ . An example of a suitable liver disease is one caused by a viral infection such as hepatitis or an autoimmune disease. Bispecific molecules targeting both activin and TGF- $\beta$  are also disclosed.

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## METHOD FOR PREVENTING OR TREATING LIVER DISEASE

### Background of the Disclosure

#### Field of the Invention

5 This invention relates to a method for preventing hepatic injury or liver cell death associated with liver disease such as hepatitis. In particular, this invention is directed to the use of an antagonist to activin to arrest or prevent death of hepatocytes that occurs with certain liver disorders.

#### Description of Background and Related Disclosures

10 Activin consists of a homodimer or heterodimer of inhibin  $\beta$  subunits, which may be  $\beta_A$  or  $\beta_B$  subunits. Vale *et al.*, Recent Prog. Horm. Res., **44**: 1-34 (1988). There is 95-100% amino acid conservation of  $\beta$  subunits among human, porcine, bovine, and rat activins. The  $\beta_A$  and  $\beta_B$  subunits within a given species are about 64-70% homologous.

The activin  $\beta_A$  and  $\beta_B$  homodimers ("Activin A" and "Activin B," respectively) have been  
15 identified in and purified from follicular fluid, and both molecules have been cloned and their genes expressed. Mason *et al.*, Biochem. Biophys. Res. Commun., **135**: 957 (1986); EP Pub. No. 222,491 published May 20, 1987; Mason *et al.*, Molecular Endocrinol., **3**: 1352-1358 (1989); Schwall *et al.*, Mol. Endocrinol., **2**: 1237-1242 (1988); Nakamura *et al.*, J. Biol. Chem., **267**: 16385-16389 (1992). The complete sequence of the  $\beta_B$  subunit is published in  
20 Serono Symposium Publications, entitled "Inhibin- Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion", eds. H.G. Burger *et al.*, abstract by A.J. Mason *et al.*, vol. 42, pp. 77-88 (Raven Press, 1987), entitled "Human Inhibin and Activin: Structure and Recombinant Expression in Mammalian Cells." The recombinant molecule has been shown to increase serum levels of FSH in rats when delivered by subcutaneous injection. Schwall  
25 *et al.*, Endocrinol., **125**: 1420-1423 (1989); Rivier and Vale, Endocrinol., **129**: 2463-2465 (1991).

Activin was initially identified in follicular fluid as a naturally occurring gonadal peptide involved in the regulation of the secretion of follicle-stimulating hormone (FSH) by rat anterior pituitary cells. Vale *et al.*, Nature, **321**: 776-779 (1986); Ling *et al.*, Nature, **321**: 779-782  
30 (1986); DePaolo *et al.*, Proc. Soc. Exp. Biol. Med., **198**: 500-512 (1991); Ying, Endocrine Rev., **9**: 267-293 (1988).

Subsequent studies revealed other activities, including the effects on follicular granulosa cell differentiation (Sugino *et al.*, Biochem. Biophys. Res. Commun., **153**: 281-288 [1988]), spermatogonial proliferation (Mather *et al.*, Endocrinol., **127**: 3206-3214 [1990]), erythroid  
35 differentiation (EP Publ. No. 210,461 published February 4, 1987 [where the protein is called BUF-3]; Eto *et al.*, Biochem. Biophys. Res. Commun., **142**: 1095-1103 [1987] and Murata *et al.*, Proc. Natl. Acad. Sci. USA, **85**: 2434-2438 [1988] [where the activin is called EDF]; Yu *et al.*, Nature, **330**: 765-767 [1987] [where the activin is called FRP]), stimulation of insulin

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secretion by pancreatic islets (Totsuka *et al.*, Biochem. Biophys. Res. Commun., **156**: 335-339 [1988]), enhancement of proliferation of fibroblast (Hedger *et al.*, Mol. Cell Endocrinol., **61**: 133-138 [1989]), stimulation of glucose production by hepatocytes (Mine *et al.*, Endocrinology, **125**: 586-591 [1989]), induction of a dose-dependent increase in inositol phosphates in rat parenchymal liver cells, an effect also seen with EGF (Mine *et al.*, Biochem. Biophys. Res. Commun., **186**: 205-210 [1992]), modulation of somatotroph functions (Billestrup *et al.*, Mol. Endocrinol., **4**: 356-362 [1990]), modulation of nerve cell differentiation (Schubert *et al.*, Nature, **344**: 868-870 [1990]; Hashimoto *et al.*, Biochem. Biophys. Res. Commun., **173**: 193-200 [1990]), and mesoderm induction. Smith *et al.*, Nature, **345**: 729-731 (1990); Mitrani *et al.*, Cell, **63**: 495-501 (1990).

It has also been found that chronic renal failure serum contains as much activin as normal serum, but the difference between normal serum and the serum of patients with renal failure exists in the context of a specific inhibitor of activin, with the suggestion that activin could be utilized in the therapy of the anemia of such patients. Shiozaki *et al.*, Biochem. Biophys. Res. Commun., **183**: 273-279 (1992). While these activities have been demonstrated *in vitro*, the role of activin *in vivo* remains poorly understood.

Inhibin and activin are members of a family of growth and differentiation factors. The prototype of this family is transforming growth factor-beta (TGF- $\beta$ ) (Derynck *et al.*, Nature, **316**: 701-705 (1985)), which, according to one source, also possesses FSH-releasing activity. Ying *et al.*, Biochem. Biophys. Res. Commun., **135**: 950-956 (1986). Other members of the TGF- $\beta$  family include the Mullerian inhibitory substance, the fly decapentaplegic gene complex, and the product of *Xenopus* Vg-1 mRNA.

A new class of gonadal protein factors, named follistatin or FSH-suppressing protein (FSP), has been isolated from side fractions derived from purifying porcine and bovine ovarian inhibins and activins. Ying, Endoc. Rev., **9**: 267-293 (1988); Ling *et al.*, "Isolation and characterization of gonadal polypeptides that regulate the secretion of follicle stimulating hormone," in Hodgen *et al.*, eds., Non-Steroidal Gonadal Factors: Physiological Roles and Possibilities in Contraceptive Development, Jones Institute Press, Virginia, (1988), pp. 30-46. Follistatin was initially characterized by its ability to suppress FSH secretion from the pituitary. The action of follistatin is apparently similar to that of inhibin, but structurally the two proteins are quite different. Ueno *et al.*, Proc. Natl. Acad. Sci. USA, **84**: 8282-8286 (1987); Robertson *et al.*, Biochem. Biophys. Res. Commun., **149**: 744-749 (1987).

Follistatin is a glycosylated single-chain protein that is found in forms having molecular weights ranging from 31 to 39 kDa. All of these forms have similar amino acid compositions and identical amino-terminal amino acid sequences. The molecular cloning of cDNA with the gene of follistatin revealed two forms, a smaller molecular weight form and a larger form, which are generated by alternative splicing. The smaller form represents a carboxy-terminal truncated form of the larger precursor.

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Recent examinations of follistatin gene expression in rat tissues have shown that follistatin mRNA is detected not only in the gonads but also in the kidney, decidual tissue, pancreas, cerebral cortex, pituitary, *etc.* Shimasaki *et al.*, Mol. Endocrinol., **3**: 651-659 (1989); Kaiser *et al.*, Endocrinology, **126**: 2768-2770 (1990); Michel *et al.*, Biochem. Biophys. Res. Comm., **173**: 401-407 (1990).

It has been found that follistatin is able to neutralize the diverse actions of activin in various systems such as stimulation of FSH secretion by cultured pituitary cells (Kogawa *et al.*, Endocrinology, **128**: 1434-1440 [1991]) and induction of mesodermal tissue formation in *Xenopus* oocytes. Asashima *et al.*, Arch. Dev. Biol., **200**: 4-7 (1991). It has been found, in fact, that immunoreactive follistatin is widespread in rat tissues, including hepatic cells, which demonstrated homogeneous immunoreactivity from moderate to strong. Kogawa *et al.*, Endocrinol. Japan, **38**: 383-391 (1991). The authors suggest that follistatin is a ubiquitous protein regulating a wide variety of activin actions.

Liver damage occurs in such clinical conditions as viral infections and autoimmune diseases. Symptoms of liver damage occurring as a result of these clinical conditions include, for example, fulminant hepatic failure, cholestasis, and liver tissue necrosis, and in many instances, improved liver function is vital to the survival of patients.

Elucidation of the regulation of liver growth has made significant progress in recent years. Specific factors have been identified that positively or negatively regulate liver cell proliferation. Cruise *et al.*, Hepatology, **7**: 1189-1194 (1987); Alison, Phys. Rev., **66**: 499-541 (1986). Included among these factors that stimulate DNA synthesis in isolated hepatocytes *in vitro* are hepatocyte growth factor (HGF) (Zarnegar and Michalopoulos, Cancer Res., **49**: 3314-3320 [1989]; Nakamura *et al.*, Nature, **342**: 440-443 [1989]), epidermal growth factor (EGF) (McGowan *et al.*, J. Cell. Physiol., **180**: 353-363 [1981]), and transforming growth factor  $\alpha$  (TGF- $\alpha$ ) (Mead and Fausto, Proc. Natl. Acad. Sci. USA, **86**: 1558-1562 [1989]), all of which stimulate cell proliferation. Vascular endothelial growth factor (VEGF), an endothelial cell mitogen, is expressed in the normal liver (Berse *et al.*, Mol. Biol. Cell, **3**: 211-220 [1992]), where it plays a role in tissue nutrition and waste removal. Growth factors, proteins with growth factor-like activities (Andus *et al.*, Hepatology, **13**: 364-375 [1991]), and therapeutics, such as tissue plasminogen activator (Baglin *et al.*, Bone Marrow Transplant **5**: 439-441 [1990]), have also been indicated in the treatment of liver damage.

On the other hand, interleukin  $1\beta$  (IL- $1\beta$ ) (Nakamura *et al.*, Exp. Cell Res., **179**: 488-497 [1988]), TGF- $\beta 1$  (Braun *et al.*, Proc. Natl. Acad. Sci. USA, **85**: 1539-1543 [1988]; Nakamura *et al.*, Biochem. Biophys. Res. Comm., **133**: 1042-1050 [1985]; Carr *et al.*, Cancer Res., **46**: 2330-2334 [1986]; Castilla *et al.*, New Eng. J. Med., **324**: 933-940 (1992); Houck *et al.*, J. Cell. Physiol., **135**: 551-555 [1988]; Strain *et al.*, Biochem. Biophys. Res. Commun., **145**: 436-442 [1987]), and activin (PCT Pub. WO92/22321 pub 23 December 1992) are known

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to inhibit hepatocyte DNA synthesis *in vitro*. TGF- $\beta$ 1 has been shown to inhibit *in vivo* DNA synthesis taking place after partial hepatectomy. Russell *et al.*, Proc. Natl. Acad. Sci. USA, 85: 5126-5130 [1988].

5 Cell proliferation, however, is not the only determinant of liver size. Many substances are cytostatic in that they inhibit mitogenesis, or cell division, but they do not kill the cell. Cell death by apoptosis was found to be an important determinant in the control of organ size under a number of different conditions. Apoptosis is a physiological cell death wherein the nucleus condenses and the cytoplasm fragments. The fragments remain intact and are phagocytosed by neighboring cells. Thus, cells dying in this manner have a characteristic  
10 morphology that differs from necrosis. Searle *et al.*, Path. Ann., 17: 229 (1982).

Apoptosis serves to eliminate excessive or unwanted cells during remodeling of embryonic tissues, during organ involution, and in regression of tumors. Wyllie *et al.*, Int. Rev. Cytol., 68: 251-306 (1980); Lockshin *et al.*, J. Gerontol., 45: B135-B140 (1990). Evidence suggests that apoptosis is under the control of growth-regulatory signals, such as  
15 hormones. Wyllie *et al.*, J. Pathol., 111: 85-94 (1973); Nawaz *et al.*, Am. J. Pathol., 27: 551-559 (1987); Kyprianou and Isaacs, Mol. Endocrinol., 3: 1515-1522 (1989); Sanford *et al.*, Pathology, 16: 406-410 (1984). In the liver apoptosis is involved in normal and pre-neoplastic cell turnover. Bursch *et al.*, Arch. Toxicol., 59: 221-227 (1986); Bursch *et al.*, Carcinogenesis, 5: 453-458 (1984); Schulte-Hermann *et al.*, Cancer Res., 50: 5127-5135  
20 (1990). Hepatomitogens and tumor promoters inhibit apoptosis, and withdrawal thereof can induce apoptosis. Wyllie *et al.*, J. Pathol., *supra*.

TGF- $\beta$ 1 appears to be a negative regulator of liver growth, and the TGF- $\beta$  molecule is associated with regression of other epithelial tissues in the embryo (Silberstein and Daniel, Science, 237: 291-293 [1987]) or adult (Kyprianou and Isaacs, *supra*) and of certain cancers.  
25 Kyprianou *et al.*, Cancer Res., 51: 162-166 (1991). Recently, it was reported that cell proliferation and apoptosis are coordinately regulated by TGF- $\beta$ 1 in cultured uterine epithelial cells. Rotello *et al.*, Proc. Natl. Acad. Sci. USA, 88: 3412-3415 (1991).

Studies *in vivo* showed that apoptotic hepatocytes in normal and preneoplastic liver exhibited immunostaining for TGF- $\beta$ 1. Oberhammer *et al.*, Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl., 343: R24 (1991). See also Oberhammer *et al.*, Cancer Res., 51: 2478-2485 (1991). Evidence has now been found that hepatocyte death induced by TGF- $\beta$ 1 *in vitro* is indeed apoptosis. Oberhammer *et al.*, Proc. Natl. Acad. Sci. USA, 89: 5408-5412 (1992).  
30 Chronic active hepatitis is associated with piecemeal necrosis, which is a term used to describe the histology of the areas of dying hepatocytes in the liver. This is described more fully in Perrillo, "Chronic Hepatitis," in Diseases of the Liver and Biliary Tract, Gary Gitnick, ed., Mosby Year Book, 1992, p. 299 *et seq.* Kerr *et al.* have proposed that piecemeal necrosis is actually apoptosis. Kerr *et al.*, The Lancet, 827-828 (1979). It may be due to an  
35 interaction between hepatocytes and immune cells, but other factors may also be involved,

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as the precise mechanisms that lead to piecemeal necrosis are not completely defined. In addition, the histopathologic terms acidophilic bodies and Councilman bodies refer to what are now known as apoptotic bodies.

5 Liver damage of this type can be diagnosed and monitored by histological evaluation of liver tissue, which is helpful in determining the type and extent of liver damage. Sinclair, Textbook of Internal Medicine, 569-575 (1992) (editor, Kelley; Publisher, J.B. Lippincott Co.; Bursch *et al.*, Carcinogenesis, 11, 847-853 (1990). It is known that results from *in vitro* biochemical tests measuring liver function or serum markers and/or results from liver tissue biopsy, correlate with *in vivo* liver damage assessment. Often, a combination of biochemical  
10 tests, tissue biopsy, patient medical history, and assessment of means inducing liver damage is used in determining the extent of liver damage.

There exists a need for an effective therapy for the prevention of liver cell death associated with liver disease or damage, which may be induced by viral infection, autoimmune disease, or elevated *in vivo* levels of hepatocytotoxic proteins. This need further exists in a  
15 patient population infected with a hepatitis virus or at risk of contracting hepatitis, as in the case of accidental or potential exposure to infected blood samples.

In many instances, the treatment of hepatitis is difficult. For example, hepatitis A, an acute illness, is treated by post-exposure prophylaxis with immune globulin; however, while it prevents or ameliorates infection in 75-80% of exposed individuals, it is not as effective in  
20 common-source outbreaks, where most exposed individuals will be past the period when prophylaxis is beneficial.

It has been found that tumor necrosis factor alpha negatively regulates hepatitis B virus gene expression in transgenic mice. Gilles *et al.*, J. Virol., 66: 3955-3960 (1992). The intracellular mechanism responsible for this effect and its pathophysiologic relevance have yet  
25 to be defined. Tested or approved treatments for chronic hepatitis B and C include use of interferon-alpha-2b. Alexander *et al.*, The Lancet, ii: 66-68 (1987); Brook *et al.*, Br. Med. J., 299: 652-656 (1989); Saracco *et al.*, Hepatology, 10: 336-341 (1989). It has also been suggested that a combination of  $\beta$ - and  $\gamma$ -interferon may be an effective therapy for chronic active hepatitis B when started early after infection. Caselmann *et al.*, Gastroenterology, 96:  
30 449-455 [1989]. However, interferon therapy is associated with side effects that prevent initiation of therapy in 10-20% of the cases. Further limitations include lack of efficacy in a large number of cases, the formation of specific antibodies, and the lack of suitable cellular receptors. Emphasis is hence placed on prophylactic measures against hepatitis, including vaccines and immunoglobulins against B virus infection and general screening and protection  
35 against C virus infection. Scalise and Corbelli, Nephron, 61: 255-257 (1992).

Hepatitis D occurs only in patients with hepatitis B, and the delta hepatitis virus responsible therefor has a direct effect on the hepatocytes. This is in contrast to the viruses causing hepatitis A and B, where the detrimental effects of the disease are attributed to the

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individual's immune response. The only known treatment for hepatitis D is prevention of hepatitis B. There is no current vaccine or treatment for hepatitis E, which occurs after natural disasters in developing regions of the world.

The various known hepatitis viruses are reviewed in Iwarson, Scand. J. Infect. Dis., 24: 129-135 (1992); Kools, Hepatitis, 91: 109-114 (1992); Consolo and Freni, Nephron, 61: 251-254 (1992); AORN Journal, 55: 790-800 (1992).

It would be particularly desirable to provide means for the prevention of liver cell death and of the further progression of liver cell death in situations where early intervention is critical. This would be particularly beneficial when known antidotes are no longer effective because of the time elapsed since the exposure to the causative factor of liver cell death.

Accordingly, it is an object of the present invention to provide means for preventing liver cell death associated with liver disorders, particularly those mediated by activin.

It is a specific object to provide means for therapeutic intervention in patients showing symptoms of liver cell death due to liver disease.

It is another specific object to provide means for prophylaxis of liver cell death in patients at risk of incurring liver cell death as a result of liver disease such as hepatitis.

These and further objects will be apparent to one of ordinary skill in the art.

#### Summary of the Invention

Accordingly, the invention concerns the use of an activin antagonist in the prevention of hepatic injury or hepatocyte death associated with certain liver disorders in patients at risk of having the disorder or having been diagnosed with the disorder.

More specifically, the present invention relates to a method for preventing hepatic injury or hepatocyte death in a mammal comprising administering to the mammal an effective amount of an activin antagonist.

In another embodiment, this invention relates to a molecule with dual specificity for activin and TGF- $\beta$  comprising a first domain having activin antagonist activity and a second domain having TGF- $\beta$  antagonist activity.

In a preferred embodiment, this molecule is a single-chain polypeptide with an activin antagonist amino acid sequence in the first domain and a TGF- $\beta$  antagonist amino acid sequence in the second domain.

In another preferred embodiment, this molecule has immunoglobulin activity. For example, it is suitably a bispecific antibody wherein the first domain is an anti-activin antibody and the second domain is an anti- $\beta$  antibody. Alternatively, it may be a bispecific immunoadhesin. In one aspect of such immunoadhesin, the first domain is an activin antagonist other than an anti-activin antibody and the second domain is an anti-TGF- $\beta$  antibody or a fusion of a TGF- $\beta$  antagonist other than an anti-TGF- $\beta$  antibody to an immunoglobulin. In another aspect of such immunoadhesin, the first domain is an anti-activin

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antibody or a fusion of an activin antagonist other than an anti-activin antibody to an immunoglobulin, and the second domain is a TGF- $\beta$  antagonist other than an anti-TGF- $\beta$  antibody.

5

### Brief Description of the Drawings

Figure 1 represents a graph of liver weight, normalized to body weight, in animals that were infused with activin (60  $\mu$ g/day) or vehicle for the number of days indicated on the x-axis. Some animals were infused for 10 days, then the pumps were removed and the animals were allowed to recover for two weeks (Recovery). Bars represent the mean  $\pm$  standard deviation of 2-8 observations.

10

Figure 2 shows the effect of activin on viability of hepatocytes as determined by metallothionine (MTT) reducing activity. Hepatocytes were cultured for 48 hours in the presence of no additions (control), activin (10 or 100 ng/ml), or an amount of acetic acid equivalent to that contained in 100 ng/ml activin. Cultures were carried out in serum-free medium. MTT reduction was then measured as an index of viability. Bars represent the mean  $\pm$  standard deviations, n=6. Asterisks indicate groups that are different from controls.

15

Figure 3 shows the time course for the effect of activin on hepatocyte viability. Hepatocytes were plated overnight in 5% fetal bovine serum. The medium was then replaced with serum-free medium containing no additions or activin at 1, 10, or 100 ng/ml. After 4, 8, 24, and 48 hours, MTT reduction was measured. Data are expressed as a percentage of control at each time point. Bars represent mean  $\pm$  standard deviation, n = 4.

20

Figure 4 represents dose-response curves for the effect of activin A (solid circles), activin B (open circles), and TGF- $\beta$  (open triangles) on hepatocyte viability. Hepatocytes were placed overnight in 5% fetal bovine serum. The medium was then replaced with serum-free medium containing no additions or the indicated concentrations of activin A, activin B, or TGF- $\beta$ . Twenty-four hours later, MTT reduction was measured. Data are expressed as percentage of control. Each point is the mean  $\pm$  standard deviation, n = 6.

25

Figure 5 shows the effect of TGF- $\beta$  on the activin bioassay. K562 cells were cultured with the indicated concentrations of activin or TGF- $\beta$ . After 4 days, hemoglobin accumulation was determined by incubating cell lysate with tetramethylbenzidine plus hydrogen peroxide and then measuring optical density at 595 nm. Each point is the mean  $\pm$  standard deviation of duplicates.

30

Figure 6 shows that there is no TGF- $\beta$ 1 immunoreactivity in the activin. The indicated concentrations of TGF- $\beta$ 1 and activin were tested for immunoactivity using a double antibody sandwich ELISA. Each point is the mean  $\pm$  standard deviation of duplicates.

35

Figure 7 shows the effect of neutralizing TGF- $\beta$  antibody (Fig. 7A), follistatin (Fig. 7B), and inhibin (Fig. 7C) on hepatocyte viability. Hepatocytes were plated overnight in 5% fetal bovine serum. The medium was then replaced with serum-free medium containing no addition

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(control), activin (10 ng/ml), or TGF- $\beta$  (1 ng/ml), each in the absence (open bar) or presence of a neutralizing antibody to TGF- $\beta$ , follistatin, or inhibin. The TGF- $\beta$  antibody was used at 250  $\mu$ g/ml (stippled bar) or 500  $\mu$ g/ml (hatched bar). Follistatin was used at a final concentration of 100 ng/ml (stippled bar) or 300 ng/ml (hatched bar). Follistatin and activin were preincubated for one hour before being fed to the cultures. The concentration of inhibin was 100 ng/ml (stippled bar) or 1000 ng/ml (hatched bar). Bars represent mean  $\pm$  standard deviation, n = 6. Asterisks indicate groups that are different from the response obtained in the absence of antibody, follistatin, or inhibin.

Figure 8 shows the reversibility of response to activin (Fig. 8A) or TGF- $\beta$  (Fig. 8B). Hepatocytes were plated overnight in 5% fetal bovine serum. At time = 0, the medium was replaced with serum-free medium containing no addition (control), activin (10 ng/ml), or TGF- $\beta$  (1 ng/ml). At 1, 2, 4, or 8 hours, cells were washed once and replenished with serum-free medium containing no additions. One group was continuously exposed to activin or TGF- $\beta$  for 24 hours. At t = 24 hours, MTT reduction was measured. Bars represent mean  $\pm$  standard deviation, n = 6.

#### Detailed Description of the Invention

##### **Definitions**

The phrase "hepatic injury or hepatocyte death" is used herein to refer to cell death in, or damage to, the liver that is characterized by apoptosis, which is a type of physiological cell death in which the nucleus of the cell condenses and the cytoplasm fragments. The hepatocyte injury or death may result, directly or indirectly, from internal or external factors or their combinations, and, if severe, can result in hepatic failure. Examples of causes of hepatocyte injury or death include, but are not limited to, illness due to an infection, such as a viral infection, *e.g.*, hepatitis, a disorder resulting from elevated *in vivo* levels of activin, or an autoimmune disease such as autoimmune liver disease and lupoid hepatitis, as described, *e.g.*, in Peters *et al.*, Hepatology, **13**: 977-994 (1991).

Hepatic injury or liver cell death can be detected by several means. Biochemical tests have been used clinically for many years as the standard measure of hepatic injury and hepatocyte death. These tests include colorimetric assays, histological tests for detection of necrosis, and use of serum enzymes as markers. See, *e.g.*, Cornelius, in Hepatotoxicology, Meeks *et al.* eds., pgs. 181-185 (1991). The importance of such tests lies in their simplicity and the fact that they are non-invasive.

One colorimetric test useful in determining if liver cell death has occurred is to measure reduction of MTT, as described by Carmichael *et al.*, Cancer Res., **47**: 936-942 (1987). In this assay, if the cell is alive, its mitochondria will take up the dye MTT, resulting in a color change from yellow to purple. If the cell is dead, no color change will result.

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Histopathological examination of the liver is the next logical step in identifying and quantitating the nature and extent of liver cell injury and/or death.

The rationale for the use of serum enzymes in assessing liver damage is that these enzymes, normally contained in the liver cells, gain entry into the general circulation when liver cells are injured. Elevated serum enzyme activity suggests necrosis and/or cholestasis. However, there are certain limitations to the use of serum enzyme levels as a single means of diagnosing liver cell injury or death. Serum enzyme levels may increase as a result of leakage from cells with altered permeability due to systemic effects of an agent rather than specific liver cell damage. Methods for performing serum enzyme analysis are known in the art and are, for example, described in Kodavanti *et al.*, in Hepatotoxicology, Meeks *et al.* eds. (1991), pp. 241-244.

Liver function tests can be performed to assess liver injury. Liver function tests include the following:

Group I assessment of hepatic clearance of organic anions, such as, bilirubin, indocyanine green (ICG), sulfobromophthalein (BSP) and bile acids;

Group II assessment of hepatic blood flow by measurements of galactose and ICG clearance; and

Group III assessment of hepatic microsomal function, through the use of the aminopyrine breath test and caffeine clearance test. For example, serum bilirubin can be measured to confirm the presence and severity of jaundice and to determine the extent of hyperbilirubinemia, as seen in parenchymal liver disease. Aminotransferase (transaminase) elevations reflect the severity of active hepatocellular damage, while alkaline phosphatase elevations are found with cholestasis and hepatic infiltrates. Isselbacher and Podolsky, in Harrison's Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1301-1308 (1991).

Because extensive liver injury may lead to decreased blood levels of albumin, prothrombin, fibrinogen, and other proteins synthesized exclusively by hepatocytes, these protein levels may be measured as indicators of liver injury. In contrast to measurements of serum enzymes, serum protein levels reflect liver synthetic function rather than just cell injury. Podolsky, in Harrison's Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1308-1311 (1991).

In many patients, computed tomography (CT), ultrasound, scintiscans, or liver biopsy may be needed to determine the nature of the liver disease. Isselbacher and Podolsky, *supra*, and Friedman and Needleman, in Harrison's Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1303-1307 (1991).

The effect of the activin antagonist on prevention of hepatic injury or hepatocyte death can also be tested *in vivo* in transgenic animal models, such as described in U.S. Pat. No. 5,087,571 issued March 22, 1988. According to a suitable protocol, transgenic animals

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subject to liver disease or liver damage are treated with the activin antagonist to be tested or activin antagonist co-administered with another therapeutic agent useful in the treatment of the disorder or disease, and sacrificed and the liver enzyme and bilirubin levels are determined. The livers are additionally observed for hepatic lesions.

5           The term "prevent" as used in the context of the present invention includes the complete or partial blocking of the occurrence of anticipated liver cell injury or death and the interruption or moderation of the progress of liver cell injury or death that has already occurred. Whereas it is foreseen that existing hepatic injury or hepatocyte death may be completely or partially reversed, this is not a requirement under this definition.

10           The term "effective amount" is used to designate an amount effective in achieving prevention as hereinabove defined.

          Patients "at risk of undergoing hepatic injury or hepatocyte death" include those patients who are anticipated to be exposed to or who have been exposed to any factor known to have the potential of inducing hepatic injury or hepatocyte death. Such factors include  
15           viruses such as hepatitis A, B, C, D, or E, genetic factors, age, sex, nutritional status, exposure to drugs and/or alcohol, and autoimmune diseases.

          For purposes herein, "activin antagonist" refers to any molecule that inhibits the activity of activin in causing hepatic injury or death of hepatocytes. As used herein, "activin" refers to homo- or heterodimers of  $\beta$  chains of inhibin, prepro forms, and pro forms, together with  
20           glycosylation variants thereof, whether in native form or synthetic or recombinant form. Activin A refers to activin with the two chains of  $\beta_A$ . Activin AB refers to activin with the chains  $\beta_A$  and  $\beta_B$ . Activin B refers to activin with the two chains of  $\beta_B$ .

          Typically the activin antagonist is a protein that binds to an active site of activin and includes, *e.g.*, follistatin as described in Esch *et al.*, Mol. Endocrinol., 1: 849-855 [(1987);  
25           Shimasaki *et al.*, Proc. Natl. Acad. Sci. USA, 85: 4218-4222 (1988); Shimasaki *et al.*, Biochem. Biophys. Res. Comm., 152: 717-723 (1988); Shimasaki *et al.*, Mol. Endocrinol., 3: 651-659 (1989); Ueno *et al.*, Proc. Natl. Acad. Sci. USA, 84: 8282 (1987); Nakamura *et al.*, Science, 247: 836 (1990); Shimonaka *et al.*, Endocrinology, 128: 3313 (1991).

          In addition, the antagonist may be a non-proteinaceous small molecule that acts as an  
30           activin antagonist. Such molecules can be screened by their ability to inhibit the action of activin in promoting liver injury or liver cell death using the assays described above and in the examples, such as the MTT assay.

          The definition of antagonist also includes an anti-activin antibody, whether polyclonal or monoclonal. Monoclonal antibodies specific for human recombinant activin A or B can be  
35           produced as described by Corrigan *et al.*, Endocrinology, 128: 1682 (1991). Briefly, inbred HPG-hypogonadal mice (Jackson Laboratories, Wilmington, MA) are hyperimmunized in the hind footpad with purified recombinant activin A, B, or AB. Cells harvested from the draining lymph nodes are then fused with the mouse myeloma line X63-Ag8.653. Kearney *et al.*, J.

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Immunol., 123: 1548 (1979). The fusions are screened for reactivity and specificity in an ELISA using recombinant human activin A, activin B, activin AB, and inhibin A as coat proteins. Wong *et al.*, Clinical Chemistry, 36: 192 (1990). Parental hybridomas that react specifically with either recombinant human activin A, B, or AB are cloned by limiting dilution.

5 Ascites fluids are produced in Balb/c nu/nu mice, and antibody is purified by protein A-sepharose affinity chromatography (Repligen Corp., Cambridge, MA) according to established procedures (Goding, J. Immunol. Meth., 20: 241 [1978]; Ey *et al.*, Immunochemistry, 15: 429 [1978]), and stored under sterile conditions in phosphate buffered saline (PBS) at 4°C. Antibodies against activin or activin peptides that may also be suitable herein, although they

10 may also cross-react with inhibin to some degree, include those described by Lofgren *et al.*, J. Immunoassay, 12: 565 (1991); Shintani *et al.*, J. Immunol. Meth., 137: 267 (1991); Groome and Lawrence, Hybridoma, 10: 309 (1991); Groome, J. Immunol. Meth., 145: 65-69 (1992); and Schwall *et al.*, Non-Radiometric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection, pp. 205-220 (Alan R. Liss, Inc., 1988).

15 Another suitable activin antagonist herein is an inhibitor of activin such as that described in Shiozaki *et al.*, *supra*, or a soluble form of an activin receptor.

Examples of suitable activin receptors include that described in U.S. Pat 5,216,126. Briefly, the receptor is described as not binding to TGF- $\beta$ , having a molecular weight on reduced 10% SDS-PAGE of 135-150 Kd, and having an N-terminal sequence of:

20 ValLeuThrGluGluThrGluIlelleMetProThrProLysProGluLeuXaaAlaXaaXaaAsn, wherein Xaa indicates an unknown amino acid. To the extent that the "activin receptor" described in Mathews and Vale, Cell, 65: 1-20 [1991] and Mathews *et al.*, Science, 255: 1702-1705 (1992) blocks activin biological activity in hepatocytes, it is included herein. Activin receptors have also been reported by Attisano *et al.*, Cell, 68: 97-108 [1992] and Kondo *et al.*,

25 Biochem. Biophys. Res. Comm., 181: 684-690 [1991].

The definition of activin antagonists also includes fragments of the above molecules that contain the active site needed to block activin activity, including F(ab) and Fc fragments of antibodies, *etc.*

#### **Modes for Carrying Out the Invention**

30 Efficacy in preventing hepatic injury and/or cell death in certain liver diseases is seen with a treatment regimen that employs an activin antagonist administered in an effective dose.

If antibodies are employed as the antagonist, they are prepared by any suitable technique. Activin or immunogenic fragments of activin may be used to induce the formation of anti-activin antibodies, which are identified by routine screening. Such antibodies may

35 either be polyclonal or monoclonal antibodies, or antigen-binding fragments of such antibodies (such as, for example, F(ab) or F(ab)<sub>2</sub> fragments). The antibodies are monovalent or polyvalent for activin. An activin antagonist or mixtures thereof or with another suitable adjuvant therapeutic agent is generally used in a single course of therapy.

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Polyclonal antibodies to activin generally are raised in animals by multiple subcutaneous (s.c.) or intraperitoneal (i.p.) injections of the activin polypeptide together with an adjuvant. It may be useful to conjugate the activin antigen polypeptide (including its chains and fragments containing the target amino acid sequence) to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups:

The route and schedule for antibody stimulation of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject, including human subjects or antibody-producing cells obtained therefrom, can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's incomplete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same activin polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are prepared by recovering immune cells--typically spleen cells or lymphocytes from lymph node tissue--from immunized animals and immortalizing the cells in conventional fashion, *e.g.*, by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Koehler and Milstein, Eur. J. Immunol., 6: 511 (1976) and also described by Hammerling *et al.*, In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

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It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody-producing cells and the myeloma be from the same species.

5 The hybrid cell lines can be maintained *in vitro* in cell culture media. The cell lines producing the antibodies can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

10 The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion- exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, *e.g.*, ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered.

15 While routinely mouse monoclonal antibodies are used, the invention is not so limited; in fact, human antibodies may be used and may prove to be preferable. Such antibodies can be obtained by using human hybridomas (Cote *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 [1985]). In fact, according to the invention, techniques developed for the production of chimeric antibodies (Morrison *et al.*, Proc. Natl. Acad. Sci., 81: 6851 [1984]; Neuberger *et al.*, Nature, 312: 604 [1984]; Takeda *et al.*, Nature, 314: 452 [1985]; EP 184,187; EP 171,496; EP 173,494; PCT WO 86/01533; Shaw *et al.*, J. Nat. Canc. Inst., 80: 1553-1559 [1988]; Morrison, Science, 229: 1202-1207 [1985]; Oi *et al.*, BioTechniques, 4: 214 [1986]) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to block activin's activity on hepatocytes) can be used; such antibodies are within the scope of this invention.

20 25 30 35 Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as F(ab) fragments), which bypass the generation of monoclonal antibodies, are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expression system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system that contains a leader sequence that causes the expressed F(ab) protein to migrate to the periplasmic space (between the bacterial cell

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membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional F(ab) fragments for those that bind the antigen. Such activin-binding molecules [F(ab) fragments with specificity for the activin polypeptide] are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

5 Typically, the activin antagonist used in the method of this invention is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of purity, with pharmaceutically acceptable carriers, *i.e.*, carriers that are non-toxic to recipients at the dosages and concentrations employed. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo  
10 *et al.* These compositions will typically contain an effective amount of the activin antagonist, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient.

The pH of the formulation depends mainly on the particular type and the concentration  
15 of antagonist, but preferably ranges anywhere from about 3 to about 8. Formulation in an acetate buffer at pH 5 is a suitable embodiment.

Compositions particularly well suited for the clinical administration of activin antagonist include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the  
20 formulation to render the formulation isotonic.

Sterility is readily accomplished by sterile filtration through (0.2 micron) membranes. Activin antagonist ordinarily will be stored as an aqueous solution, although lyophilized formulations for reconstitution are acceptable.

The antagonist composition will be formulated, dosed, and administered in a fashion  
25 consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of activin antagonist to be  
30 administered will be governed by such considerations, and is the minimum amount necessary to prevent hepatic injury or hepatocyte death. Such amount is preferably below the amount that is toxic to the mammal or renders the mammal significantly more susceptible to infections.

As a general proposition, the pharmaceutically effective amount of the activin  
35 antagonist administered parenterally per dose will be in the range of about 0.01 to 100 mg/kg of patient body weight per day, with the typical range of activin antagonist used being about 0.1 to 50 mg/kg/day. A typical effective dose in rat experiments is given below in the examples. Interspecies scaling of dosages can be performed in a manner known in the art,

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*e.g.*, as disclosed in Mordenti *et al.*, Pharmaceut. Res., 8: 1351 (1991) and in the references cited therein.

As noted above, however, these suggested amounts of activin antagonist are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above.

The activin antagonist is administered to a subject mammal by any suitable means, including parenteral, subcutaneous, and intranasal, and, if desired, intralesional administration. Examples of parenteral administration routes include intravenous, intrapulmonary, intraarterial, intramuscular, and intraperitoneal administration. Administration may be continuous or bolus dosing in sufficient amounts to maintain therapeutically effective levels. In addition, the activin antagonist is suitably administered by pulse infusion. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

The activin antagonist need not be, but is optionally, combined or formulated with one or more therapies or vaccines or other prophylactics currently used to prevent or treat the disorder in question. Examples of these therapies include the use of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) or other growth factors, proteins with growth factor-like activities, such as tissue plasminogen activator or other therapeutics, interferon-alpha-2 $\beta$ , vaccines such as hepatitis B vaccine or immune globulins, or an antagonist to TGF- $\beta$ .

Examples of TGF- $\beta$  antagonists include antibodies to TGF- $\beta$  such as those described in Lucas *et al.*, J. Immunol., 145: 1415-1422 (1990); Dasch *et al.*, J. Immunol., 142: 1536-1541 (1989); Ellingsworth *et al.*, J. Biol. Chem., 261: 12362-12367 (1986); Cheifetz *et al.*, Cell, 48: 409-415 (1987); Florini *et al.*, J. Biol. Chem., 261, 16509-16513 (1986); Roberts *et al.*, Proc. Natl. Acad. Sci. USA, 83: 4167-4171 (1986); Assoian and Sporn, J. Cell Biol., 102: 12178-1223 (1986); Ellingsworth *et al.*, Cell. Immunol., 114: 41 (1988); Flanders *et al.*, Biochemistry, 27: 739 (1988); Keski-Oja *et al.*, Cancer Res., 47: 6451 (1988); Danielpour and Sporn, J. Cell Biochem., 13B: 84 (1989); and Danielpour *et al.*, J. Cell Physiol., 138: 79-86 (1989).

Additional TGF- $\beta$  antagonists that are suitable include non-proteinaceous small molecules that act as a TGF- $\beta$  antagonist in blocking the ability of TGF- $\beta$  to cause hepatic injury or hepatocyte death, screened by, *e.g.*, the MTT test, and a soluble form of the TGF- $\beta$  receptor or TGF- $\beta$  binding protein of any type, as described, for example, in Lin *et al.*, Cell, 68: 775-785 (1992); Lin *et al.*, J. Cell Biochem. Suppl., 16 Part B, p. 125 (1992); Wang *et al.*, Cell, 67: 797-805 (1991); EP 369,861 published 23 May 1990; Wang *et al.*, J. Cell Biochem. Suppl., 16, part B, p. 129 (1992); Lopez-Casillas *et al.*, Cell, 67: 785-795 (1991); O'Grady *et al.*, J. Biol. Chem., 266: 8583-8589 (1991); Segarini *et al.*, J. Biol. Chem., 267: 1048-1053 (1992); MacKay *et al.*, J. Biol. Chem., 265: 9351-9356 (1990); Cheifetz and

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Massague, J. Biol. Chem., 266: 20767-20772 (1991); Cheifetz and Massague, J. Cell Biochem. Suppl., 16, part B, p. 121 (1992); Ichijo *et al.*, J. Biol. Chem., 266: 22459-22464 (1991); Borisuth *et al.*, Invest. Ophthal. and Vis. Sci., 33: 596-603 (1992); Mitchell and O'Connor-McCourt, J. Cell Biol., 115: 3, Part 2, p. 265A (1991).

- 5 For recent reviews of TGF- $\beta$  receptors, see Segarini, "TGF- $\beta$  Receptors," Clinical Applications of TGF- $\beta$  (Wiley, Chichester [Ciba Foundation Symposium 157], p. 29-50, 1991), and Massague *et al.*, Annals NY Acad. Sci., p. 59-72, 1990.

- 10 The effective amount of such other agents depends mainly on the amount and type of activin antagonist present in the formulation, the type of disorder or treatment, the mode, scheduling, and regimen for administering the agents, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages. The activin antagonist and other agent may be formulated together in a single composition comprising therapeutically effective amounts of each of the agents in a pharmaceutical carrier having the  
15 appropriate pH for effective administration to the patient. Respective formulations of activin antagonist and other agent may be combined *in vitro* before administration or separately administered simultaneously, or in tandem, in either order, with any second administration taking place preferably within about 1-24 hours of the first administration, more preferably within about 1-5 hours.

- 20 The invention herein also encompasses "molecules with dual specificity for activin and TGF- $\beta$ ," which would include bispecific antibodies/immunoadhesins and bispecific linear molecules, such as the so-called "Janusin" structures recently reported by Traunecker *et al.*, EMBO, 10: 3655-3659 (1991). Such molecule with dual specificity for activin and TGF- $\beta$  would comprise a domain having activin antagonist activity and a domain having TGF- $\beta$  antagonist activity. In one embodiment the molecule is a single-chain polypeptide with an  
25 activin antagonist amino acid sequence in one domain and a TGF- $\beta$  antagonist amino acid sequence in the other domain.

- For purposes herein, immunoadhesins are antibody-like molecules that combine the binding specificity of a protein such as a cell-surface receptor, a cell-adhesion molecule or a  
30 ligand (an "adhesin"), with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity that is other than the antigen recognition and binding site (antigen-combining site) of an antibody (*i.e.*, is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous  
35 amino acid sequence comprising at least the binding domain of a receptor (including cell adhesion molecules) or a ligand.

Immunoadhesins can possess many of the valuable chemical and biological properties of human antibodies. Since immunoadhesins can be constructed from a human protein

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sequence with a desired specificity linked to an appropriate human immunoglobulin hinge and constant domain (Fc) sequence, the binding specificity of interest can be achieved using entirely human components. Such immunoadhesins are minimally immunogenic to the patient, and are safe for chronic or repeated use.

5           The immunoglobulin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA, IgE, IgD or IgM. The antagonist portion of the sequence is preferably fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2, and CH3 domains of an IgG<sub>1</sub>, IgG<sub>2</sub> or IgG<sub>3</sub> heavy chain.

10           The precise site at which the fusion for the immunoadhesin is made is not critical; particular sites are well known, can be determined by routine experimentation, and may be selected in order to optimize the biological activity, secretion or binding characteristics of the immunoadhesin.

15           Immunoadhesins reported in the literature include fusions of the T cell receptor\* (Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA, 84: 2936-2940 [1987]); CD4\* (Capon *et al.*, Nature, 337: 525-531 [1989]; Traunecker *et al.*, Nature, 339: 68-70 [1989]; Zettmeissl *et al.*, DNA Cell Biol. USA, 9: 347-353 [1990]; Byrn *et al.*, Nature, 344: 667-670 [1990]); L-selectin (homing receptor) (Watson *et al.*, J. Cell. Biol., 110: 2221-2229 [1990]; Watson *et al.*, Nature, 349: 164-167 [1991]); CD44\* (Aruffo *et al.*, Cell, 61: 1303-1313 [1990]); CD28\* and B7\* (Linsley *et al.*, J. Exp. Med., 173: 721-730 [1991]); CTLA-4\* (Lisley *et al.*, J. Exp. Med., 174: 561-569 [1991]); CD22\* (Stamenkovic *et al.*, Cell, 66: 1133-1144 [1991]); TNF receptor (Ashkenazi *et al.*, Proc. Natl. Acad. Sci. USA, 88: 10535-10539 [1991]; Lesslauer *et al.*, Eur. J. Immunol., 27: 2883-2886 [1991]; Peppel *et al.*, J. Exp. Med., 174: 1483-1489 [1991]); NP receptors (Bennett *et al.*, J. Biol. Chem., 266: 23060-23067 [1991]); and IgE receptor  $\alpha$ -chain\* (Ridgway and Gorman, J. Cell. Biol., 115: abstr. 1448 [1991]), where the asterisk (\*) indicates that the receptor is a member of the immunoglobulin superfamily.

25           If the two arms of the antibody-like immunoadhesin structure have two different specificities, the immunoadhesin is referred to as bispecific using the analogy of bispecific antibodies. In the present invention, one example is where one arm (or domain) of the antibody-like, bispecific immunoadhesin structure is comprised of an activin antagonist (excluding an anti-activin antibody) such as a small molecule, follistatin, or an activin receptor, and the second arm (or domain) is comprised of either an anti-TGF- $\beta$  antibody or a fusion of a TGF- $\beta$  antagonist (other than an anti-TGF- $\beta$  antibody) to an immunoglobulin.

30           In another example, one arm of the immunoadhesin structure is comprised of a TGF- $\beta$  antagonist (excluding an anti-TGF- $\beta$  antibody) such as a small molecule or a TGF- $\beta$  receptor, and the second arm is comprised of either an anti-activin antibody or a fusion of an activin antagonist (other than an anti-activin antibody) to an immunoglobulin.

          Thus, as used herein the phrase "bispecific immunoadhesin" designates immunoadhesins (as hereinabove defined) having at least two binding specificities, one of

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which may be (but does not need to be) an antigen-binding site of an antibody. Bispecific immunoadhesins can generally be assembled as hetero-multimers, and particularly as hetero-dimers, -trimers or -tetramers, essentially as disclosed in WO 89/02922 (published 6 April 1989), in EP 314,317 (published 3 May 1989), in WO 91/08298 (published 13 June 1991),  
5 and in U.S. Patent No. 5,116,964 issued 2 May 1992.

An example of a bispecific antibody herein is one comprising a fusion of an anti-activin antibody and an anti-TGF- $\beta$  antibody.

The recombinant production of bispecific immunoadhesins and antibodies is usually based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the  
10 two heavy chains have different specificities. Milstein and Cuello, Nature, 305: 537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which the one having the correct bispecific structure needs to be isolated and purified.

Bispecific antibodies can be prepared by the so-called transfectoma method, essentially  
15 as described by Morrison, Science, 229: 1202-1207 (1985). This method is also suitable for the production of bispecific immunoadhesins, when a vector comprising the coding sequence of a chimeric (fusion) protein with a desired binding specificity is transfected into a hybridoma secreting an antibody providing the second specificity. See also Berg *et al.*, Proc. Natl. Acad. Sci. USA, 88: 4723 (1991).

20 Trimeric bispecific immunoadhesins composed of a hybrid immunoglobulin heavy chain in one arm and a hybrid immunoglobulin heavy chain-light chain pair in the other arm are prepared. These immunoadhesins are preferably produced by individually introducing into suitable host cells the DNA sequences encoding the three immunoglobulin chains making up the trimeric molecule. As a result, the ratios of these DNA sequences can be freely changed.  
25 Notwithstanding the absence of the light chain in one arm and the asymmetric structure of the trimeric molecule, these molecules can be efficiently secreted in the form of correctly assembled and folded hetero-trimers. It was further found that the asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the  
30 bispecific molecule provides for a facile way of separation.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention.

### **EXAMPLE I**

#### **35 Materials**

The recombinant forms of human activin A, human activin B, and human inhibin A employed herein are described in U.S. Pat. No. 4,798,885 issued 17 January 1989, wherein inhibin A is a dimer of the  $\alpha$  and  $\beta_A$  chains of inhibin. They are also available from Genentech,

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Inc., South San Francisco, CA as pre-clinical research reagents. The purified recombinant human follistatin was obtained by standard methods of recombinant expression as described by Esch *et al.*, Mol. Endocrinol., **1**: 849-855 [(1987); Shimasaki *et al.*, Proc. Natl. Acad. Sci. USA, **85**: 4218-4222 (1988). All are produced as sterile, endotoxin-free reagents.

5           The neutralizing TGF- $\beta$  monoclonal antibody 2G7 employed was deposited in the American Type Culture Collection under the ATCC Accession No. HB10240 on September 28, 1989. The antibody is also described in Lucas *et al.*, *supra*.

#### In Vivo Studies

10           Activin A or the appropriate vehicle (see below) was loaded into osmotic mini-pumps that were then primed overnight in saline and implanted subcutaneously in female Sprague-Dawley rats (100-120 g). The animals were anesthetized with ketamine (50 mg/kg)-xylazine (10 mg/kg) intraperitoneally during pump implantation and removal. All procedures were performed in accordance with the NIH Guidelines.

15           Alza™ 2ML1 pumps (Alza Corporation, Palo Alto, CA) were used for infusions lasting 1, 3, and 5 days, while Alza™ 2ML2 pumps were used for infusions longer than five days. The concentration of activin was adjusted to deliver 60  $\mu$ g/day. Two different lots of activin were used with similar results. The first was formulated in 10 mM acetic acid, the second in 50 mM Tris (pH 7.5), 150 mM NaCl. The appropriate vehicle control (buffer only) was used for each experiment.

20           After 1, 3, 5, and 10 days of infusion, rats were anesthetized with ketamine-xylazine, and blood was collected by cardiac puncture and allowed to clot for one hour at room temperature. Serum aliquots were stored at -70°C prior to measurement of bilirubin, ALT, and AST on a Monarch Model 7000 automated analyzer. The liver was dissected free from connective tissue and weighed, and pieces were fixed in neutral buffer formalin. Paraffin-  
25           embedded sections were cut at 4  $\mu$ m stained with hematoxylin and eosin.

          In some animals that had been infused for 10 days, the pumps were removed. Two weeks later, serum and livers were collected as described above.

#### In Vitro Studies

30           Hepatocyte Cultures Hepatocytes were obtained from adult female Sprague-Dawley rats by collagenase perfusion, as described by Garrison and Haynes, J. Biol. Chem., **250**: 2769-2777 (1975). The cells were cultured in William's E medium supplemented with penicillin (100 U/ml), streptomycin sulfate (100  $\mu$ g/ml), L-glutamine (2 mM), transferrin (10  $\mu$ g/ml), and trace elements (0.01%) in 96-well plates (Falcon). The cells were cultured at 37°C in 5% CO<sub>2</sub>.

35           Measurement of MTT Reduction Viability was assessed by measuring the reduction of MTT, essentially as described by Carmichael *et al.*, Cancer Res., **47**: 936-942 (1987). Collagenase-dispersed hepatocytes were plated at a density of 4000 cells/well in 96-well plates, as described above. In the initial experiments, cells were plated and cultured in serum-

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free medium. In subsequent experiments, however, it was found useful to plate the cells overnight in medium containing 5% fetal bovine serum, after which the treatments were conducted in serum-free medium. At the end of the incubation, 5  $\mu$ l of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well and incubated at 37°C for four hours.

5 The media was removed by gently inverting the plate and blotting on a paper towel. The cells were solubilized by addition of 100  $\mu$ l DMSO followed by shaking for five minutes on an orbital shaker.

10 The absorbance at 560 nm, less the absorbance at the reference wavelength of 690 nm, was measured in an automatic plate reader (SLT Labinstruments). In some experiments, cells were cultured in more than one microtiter plate. In those cases, data were normalized to the controls within each plate.

Activin Bioassay Activin bioactivity was assessed by measuring hemoglobin accumulation in K562 cells, taking advantage of the pseudoperoxidase activity of hemoglobin. The method is a microtiter plate adaptation of that described by Clarke *et al.*, Blood, 60: 346-351 (1982). K562 cells are propagated by twice-weekly passage in RPMI containing antibiotics and 10% fetal bovine serum. For bioassay, the cells are seeded into round-bottom 96-well plates (Falcon) at a density of 12,500 per well, using the same culture medium. The factor to be tested is added at the time of seeding and the cells are incubated for 4 days at 37°C in 5% CO<sub>2</sub>. The plate is then centrifuged at 1400 rpm for 10 minutes in a Beckman table top centrifuge. The medium is removed by gently inverting the plate and blotting on absorbent paper. To each well is added 150  $\mu$ l tetramethyl benzidine (5 mg/ml in 90% acetic acid) followed by 50  $\mu$ l 0.3% H<sub>2</sub>O<sub>2</sub>. The plate is incubated in the dark for 90 minutes and absorbance at 595 nm is measured in an automatic plate reader.

20 TGF- $\beta$  ELISA TGF- $\beta$  was measured in a double-antibody ELISA that is specific for TGF- $\beta$ 1. Lucas *et al.*, *supra*.

#### Analysis of Data

Results were analyzed for analysis of variance followed by Duncan's multiple range test. The figures show data from representative experiments, each of which was repeated 3-5 times.

30

## RESULTS

### Effects of Activin *in vivo*

Liver weight rapidly decreased to 68% of controls within 1 day of activin infusion (Figure 1). It fell further to 44% of controls by day 3 and then remained stable through day 10. On gross examination, the livers from activin-treated animals were small but exhibited no indications of infarction or necrosis.

On histopathological examination, however, extensive cell death was observed surrounding the central vein. The areas of cell death were characterized by disruption of

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hepatic plates due to breakdown of intercellular attachments and individualization of degenerating hepatocytes. Occasionally, dissociated degenerating hepatocytes were observed in the lumen of the central vein. The nucleus of degenerating hepatocytes was condensed and subsequently underwent fragmentation. The cell membrane remained intact and the cytoplasm was shrunken and deeply eosinophilic. Frequently these degenerating hepatocytes, which resembled apoptotic bodies, were phagocytosed by adjacent hepatocytes. There was no leukocytic infiltration into the areas of cell death at any time point examined.

Consistent with the changes in liver weight, the extent of cell death was greater on day 3 than day 1. By day 5, numerous mitotic figures could be seen in the hepatocytes surrounding the areas of cell death. The histological changes were associated with elevations in serum levels of liver enzyme markers and bilirubin. See Table I, where serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were measured after continuous infusion of activin or vehicle for the indicated number of days.

To determine whether the effects of activin were reversible, the pumps were removed from some animals that had been infused for 10 days and the animals were allowed to recover for two weeks (2 Wk Recov.). Data are mean  $\pm$  standard deviation of 2-8 observations. At this time, liver weight was restored (Figure 1) and the histological appearance was normal. In addition, bilirubin, ALT, and AST returned to the normal range. Table I.

TABLE I

Effect of Activin Infusion on Serum Enzymes and Bilirubin

Marker	Day	Experiment 1		Experiment 2	
		Vehicle	Activin	Vehicle	Activin
ALT (IU/L)	1	28.2 $\pm$ 2.5	56.3 $\pm$ 8.5 <sup>a</sup>	nd	nd
	3	29.0 $\pm$ 4.9	53.0 $\pm$ 5.3 <sup>a</sup>	33.5 $\pm$ 6.5	39.8 $\pm$ 15.8
	5	30.8 $\pm$ 3.5	50.2 $\pm$ 8.9 <sup>a</sup>	33.3 $\pm$ 5.0	64.5 $\pm$ 12.6 <sup>a</sup>
	10	nd	nd	28.4 $\pm$ 1.8	160.0 $\pm$ 131.5 <sup>a</sup>
	2 Wk Recov	nd	nd	32.5 $\pm$ 7.1	31.0 $\pm$ 1.4
AST (IU/L)	1	48.0 $\pm$ 10.1	237.3 $\pm$ 32.3 <sup>a</sup>	nd	nd
	3	56.2 $\pm$ 13.9	180.0 $\pm$ 16.5 <sup>a</sup>	64.0 $\pm$ 9.7	147.0 $\pm$ 22.3 <sup>a</sup>
	5	53.2 $\pm$ 7.1	167.5 $\pm$ 20.3 <sup>a</sup>	64.8 $\pm$ 8.4	281.0 $\pm$ 117.5 <sup>a</sup>
	10	nd	nd	63.8 $\pm$ 6.1	726.0 $\pm$ 619.4 <sup>a</sup>
	2 Wk Recov	nd	nd	66.3 $\pm$ 13.7	59.0 $\pm$ 8.5
Bilirubin (mg/dl)	1	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2	nd	nd
	3	0.2 $\pm$ 0.1	1.7 $\pm$ 0.2 <sup>a</sup>	0.2 $\pm$ 0.1	1.6 $\pm$ 0.7 <sup>a</sup>

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5	0.2±0.1	1.4±0.7 <sup>a</sup>	0.2±0.1	4.5±0.1 <sup>a</sup>
10	nd	nd	0.1±0.1	8.6 <sup>a,b</sup>
2 Wk Recov	nd	nd	0.2±0.1	0.2±0.1

5 nd = not determined

<sup>a</sup>P<0.05 vs. vehicle control

<sup>b</sup>n = 1

#### Effects of Activin *in vitro*

10 To determine whether activin had a direct effect on the liver, it was tested on hepatocytes in primary culture. Many of the hepatocytes that had been exposed to activin appeared to be fragmenting and forming numerous cytoplasmic blebs. A few fragmenting cells could be found in control cultures, but in the activin-treated cultures, the majority of cells were undergoing fragmentation and the extent of fragmentation was much more severe.

15 Cellular ghosts were seen in almost every activin-treated culture, although only a few were present in each.

To quantitate cell viability, reduction of MTT was used as described above. In this assay, viable cells metabolize MTT, producing an insoluble formazan deposit within the cell. The cells are then solubilized and the amount of formazan can be measured

20 spectrophotometrically. Figure 2 shows the results obtained when this assay was applied to hepatocytes cultured with activin. MTT reduction was decreased by ~80% in cells exposed to activin at 10 or 100 ng/ml. Activin has limited solubility at neutral pH and stock solutions are stored in dilute acetic acid, but this diluent had no effect on the cell viability.

Although serum blocked the response, cells cultured in serum-free medium attached

25 poorly to the culture plate. It was subsequently found that activin responses could be observed if the wells were plated overnight in 5% fetal bovine serum (FBS) and then switched to serum-free medium. All of the data below were generated using cells cultured in this manner.

Figure 3 shows a time-course for the decrease in MTT reducing activity as a functional

30 of activin concentration. A small response to activin was detected as early as 8 hours, and a more significant decrease was observed at 24 and 48 hours. At the 24- and 48-hour time-points, a slight decrease in viability was observed with 1 ng/ml activin and maximal response occurred with 10 ng/ml.

Because TGF- $\beta$  influences hepatocyte mitosis and viability, as discussed above, the

35 effects of TGF- $\beta$  and activin on hepatocyte viability were compared using the MTT assay. Figure 4 contains dose-response curves for activin A, activin B, and TGF- $\beta$ 1. Consistent with the data presented above, activin caused a maximal response at 10 ng/ml.

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TGF- $\beta$  also decreased MTT reducing activity, but at roughly one-tenth of the concentration. This raised the possibility that the effects of activin may be due to a small amount of TGF- $\beta$  contamination. Therefore, both the activin and TGF- $\beta$  were tested in the K562 bioassay described above. As seen from Figure 5 TGF- $\beta$  was inactive in inducing erythroid differentiation in the K562 cells. Conversely, there was no detectable TGF- $\beta$ 1 immunoactivity in the activin preparation (Figure 6). Furthermore, a monoclonal antibody (2G7) that neutralizes the activity of TGF- $\beta$ 1, 2, and 3 (Lucas *et al.*, *supra*) blocked the response to TGF- $\beta$  but had no effect on the response to activin (Figure 7A).

Conversely, follistatin, which is an activin-binding protein (DePaolo *et al.*, *supra*), blocked the response to activin but had no effect on the response to TGF- $\beta$  (Figure 7B).

Inhibin, which antagonizes the effects of activin in many systems, had only a partial effect on the response to activin in this system, even when the inhibin concentration was 1000 ng/ml (Figure 7C). Since inhibin had only a partial effect on the hepatocyte response to activin, even when used at large doses, its ability to antagonize activin appears to be tissue specific. Inhibin had no effect on the response to TGF- $\beta$ .

To determine whether activin or TGF- $\beta$  needed to be continually present to elicit a response, cells were exposed either for 24 hours or for the first 1, 2, 4, or 8 hours of culture. MTT reduction was then measured at 24 hours. As shown in Figure 8, maximal response to activin required continuous exposure for 24 hours, although partial responses were observed in cells exposed during only the first 4-8 hours. In contrast, exposure of cells to TGF- $\beta$  for as little as one hour elicited maximal responses.

In conclusion, it is seen that infusion of activin reduced liver mass. The regressed livers showed no gross signs of infarction or necrosis, but histological examination revealed extensive cell death. Thus, exposure to elevated concentrations of activin may lead to death of hepatocytes. Because the morphology of dying cells in activin-treated livers resembles that seen in pathological conditions (historically referred to as Councilman bodies or acidophilic bodies), activin may be a causative factor in certain pathological conditions of the liver.

Consistent with the *in vivo* findings, exposure to activin *in vitro* had a direct effect on hepatocyte viability. This effect was blocked by the activin antagonist follistatin. By extrapolation to the *in vivo* findings, activin antagonists are expected to prevent hepatic injury or hepatocyte death that results from exposure to elevated activin concentrations *in vivo*.

It is seen from this data that activin B also induces cell death of hepatocytes in the rat model described above. Thus, antagonists to activin B and activin AB would be expected also to work in the treatment described herein.

It would be reasonably expected that the rat data herein may be extrapolated to horses, cows, and other mammals, correcting for the body weight of the mammal in accordance with recognized veterinary and clinical procedures. Using standard protocols and procedures, the veterinarian or clinician will be able to adjust the doses, scheduling, and mode of

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administration of the activin antagonist to achieve maximal effects in the desired mammal being treated. Humans are believed to respond in this manner as well.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

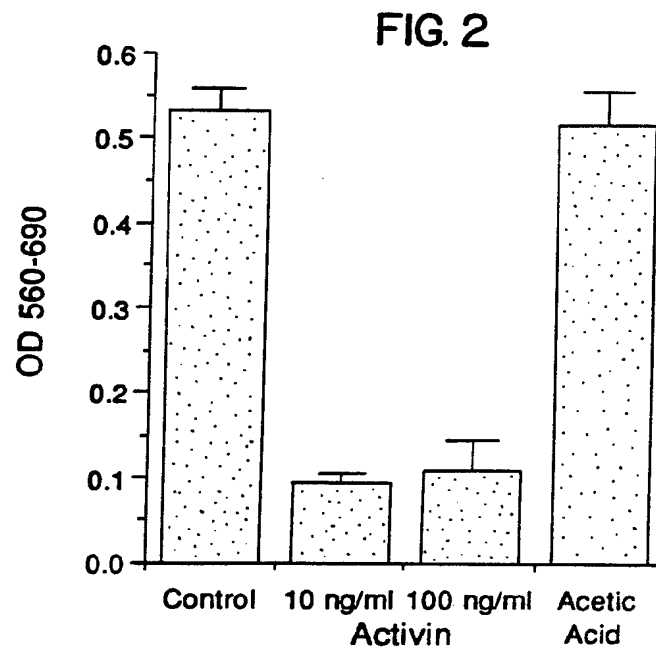
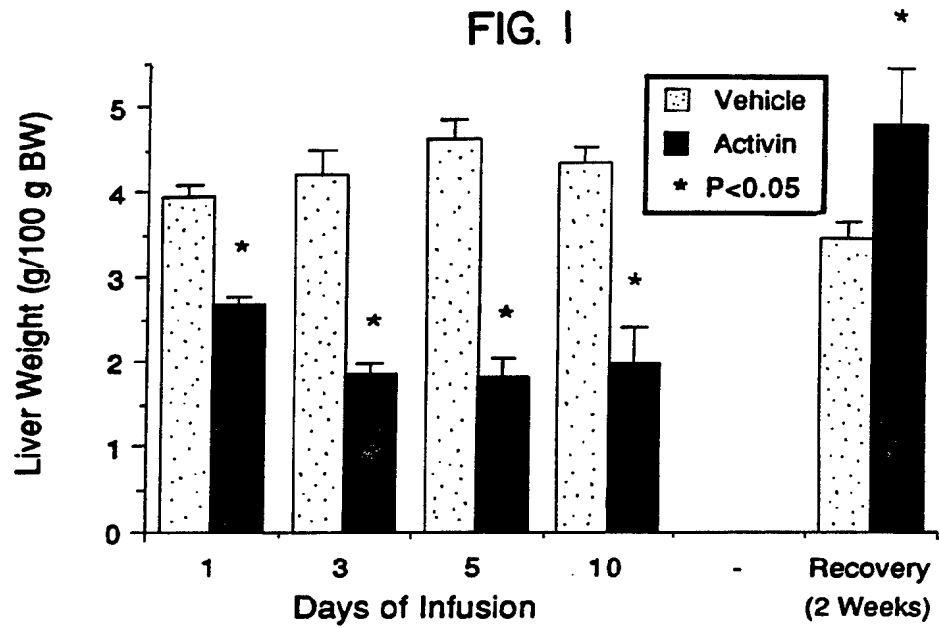
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WHAT IS CLAIMED IS:

1. A method for preventing hepatic injury or hepatocyte death in a mammal comprising administering to the mammal an effective amount of an activin antagonist.
- 5 2. The method of claim 1 wherein the hepatic injury or hepatocyte death is due to a viral infection.
3. The method of claim 2 wherein the viral infection is hepatitis.
4. The method of claim 1 wherein the hepatic injury or hepatocyte death is due to cirrhosis.
- 10 5. The method of claim 1 wherein the mammal is a human.
6. The method of claim 1 wherein the activin antagonist is follistatin.
7. The method of claim 1 wherein the activin antagonist is an anti-activin antibody.
8. The method of claim 1 wherein the activin antagonist is a soluble form of the activin receptor.
- 15 9. The method of claim 1 further comprising administering to the mammal an effective amount of a TGF- $\beta$  antagonist.
10. The method of claim 9 wherein the TGF- $\beta$  antagonist is an anti-TGF- $\beta$  antibody.
11. The method of claim 9 wherein the TGF- $\beta$  antagonist is a soluble form of the TGF- $\beta$  receptor.
- 20 12. A molecule with dual specificity for activin and TGF- $\beta$  comprising a first domain having activin antagonist activity and a second domain having TGF- $\beta$  antagonist activity.
13. The molecule of claim 12 that is a single-chain polypeptide with an activin antagonist amino acid sequence in the first domain and a TGF- $\beta$  antagonist amino acid sequence in the second domain.
- 25 14. The molecule of claim 12 that has immunoglobulin activity.
15. The molecule of claim 14 that is a bispecific antibody wherein the first domain is an anti-activin antibody and the second domain is an anti- $\beta$  antibody.
16. The molecule of claim 14 that is a bispecific immunoadhesin wherein the first domain is an activin antagonist other than an anti-activin antibody and the second domain is an anti-TGF- $\beta$  antibody or a fusion of a TGF- $\beta$  antagonist other than an anti-TGF- $\beta$  antibody to an immunoglobulin.
- 30 17. The molecule of claim 14 that is a bispecific immunoadhesin wherein the first domain is an anti-activin antibody or a fusion of an activin antagonist other than an anti-activin antibody to an immunoglobulin, and the second domain is a TGF- $\beta$  antagonist other than an anti-TGF- $\beta$  antibody.
- 35



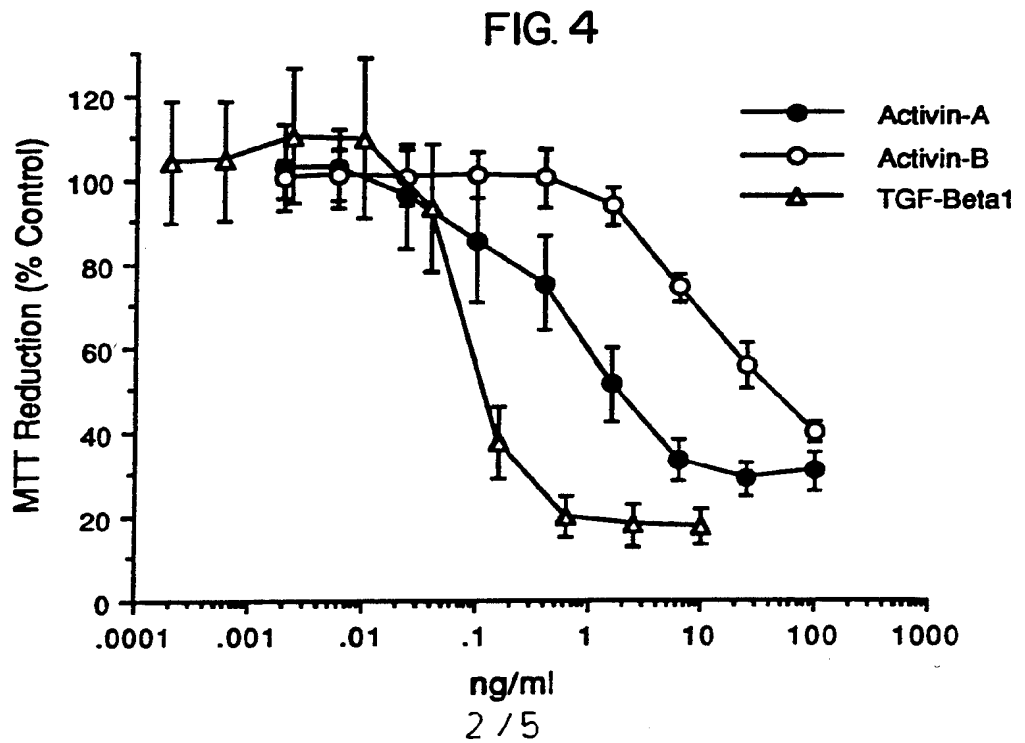
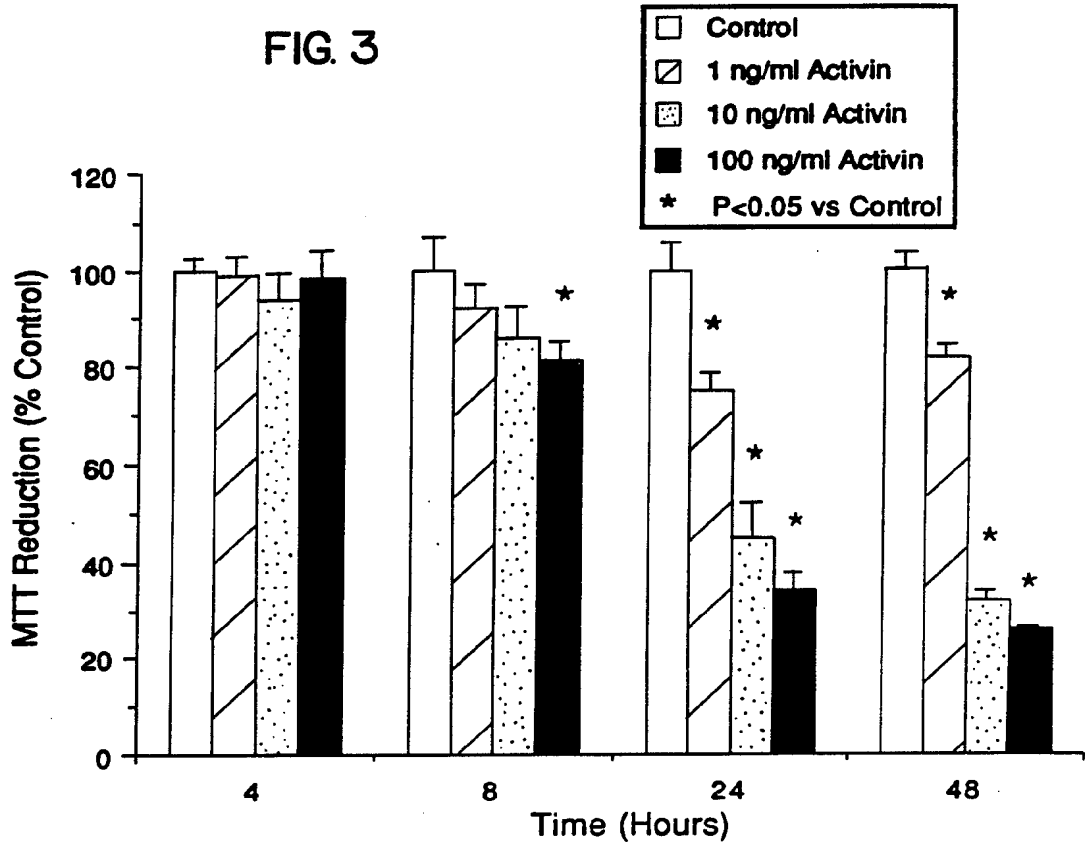


FIG. 5

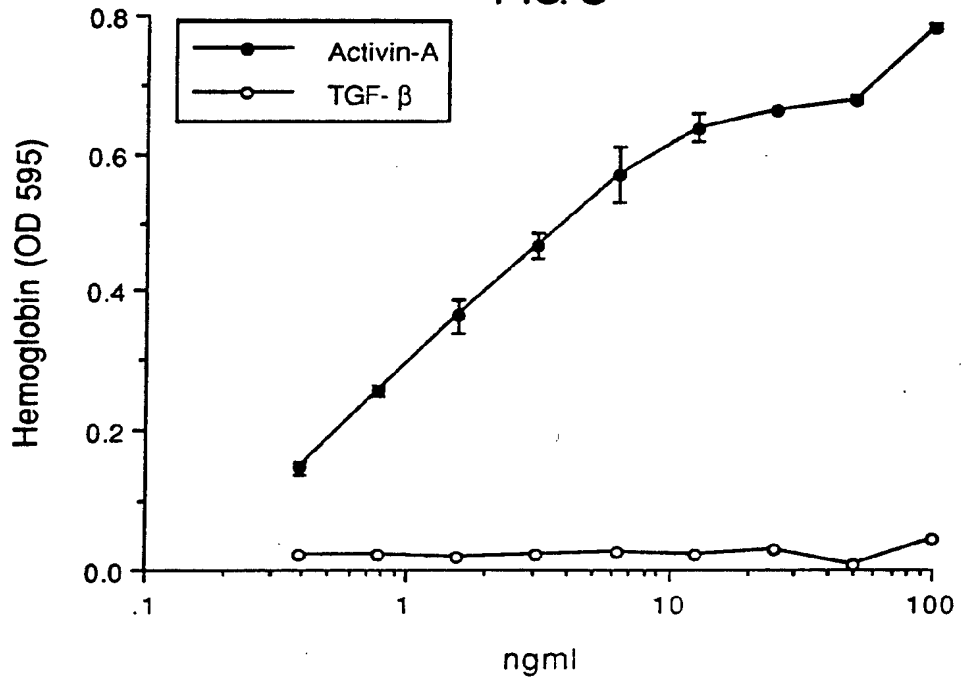
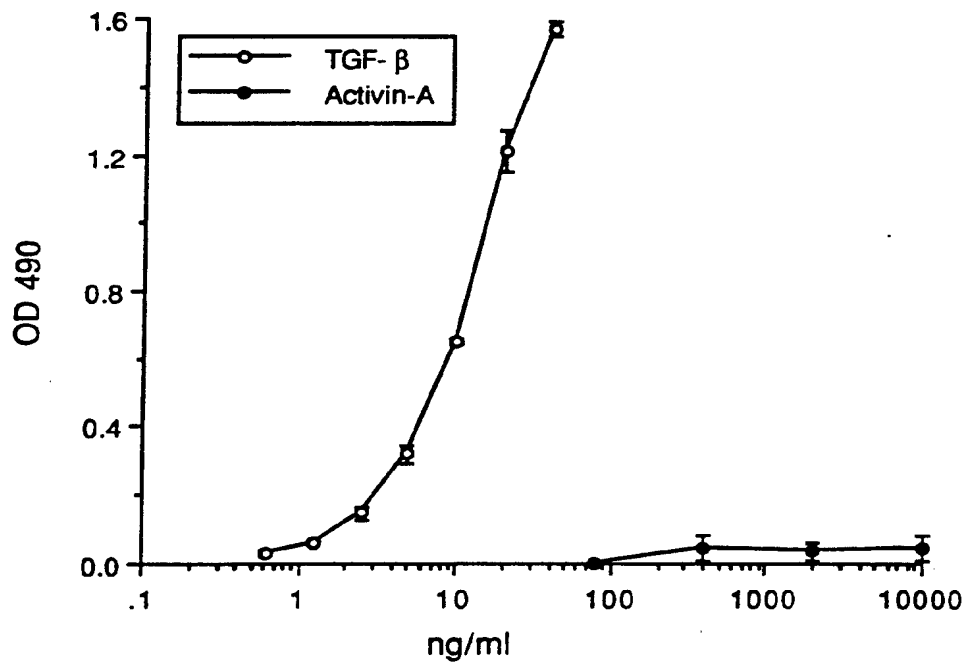


FIG. 6



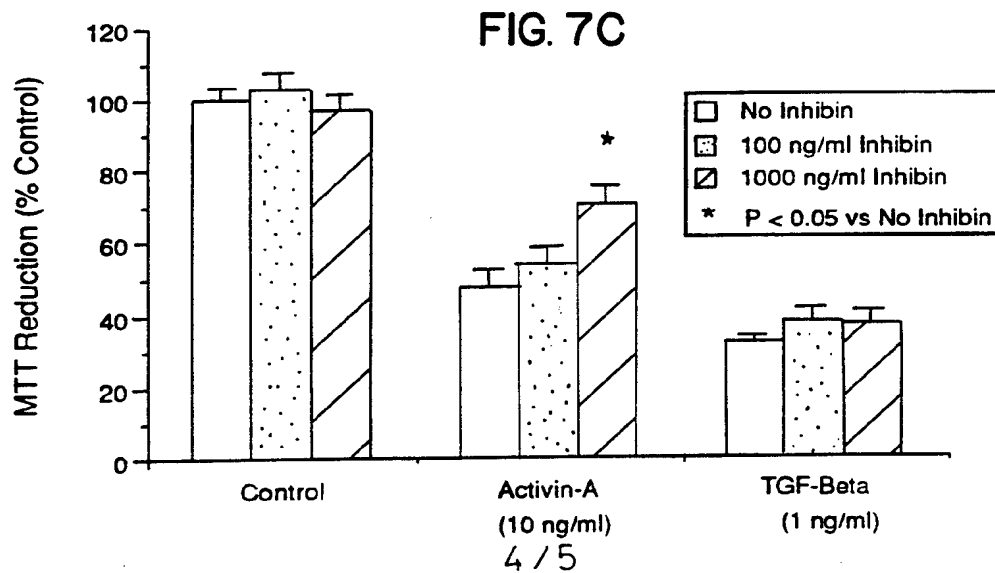
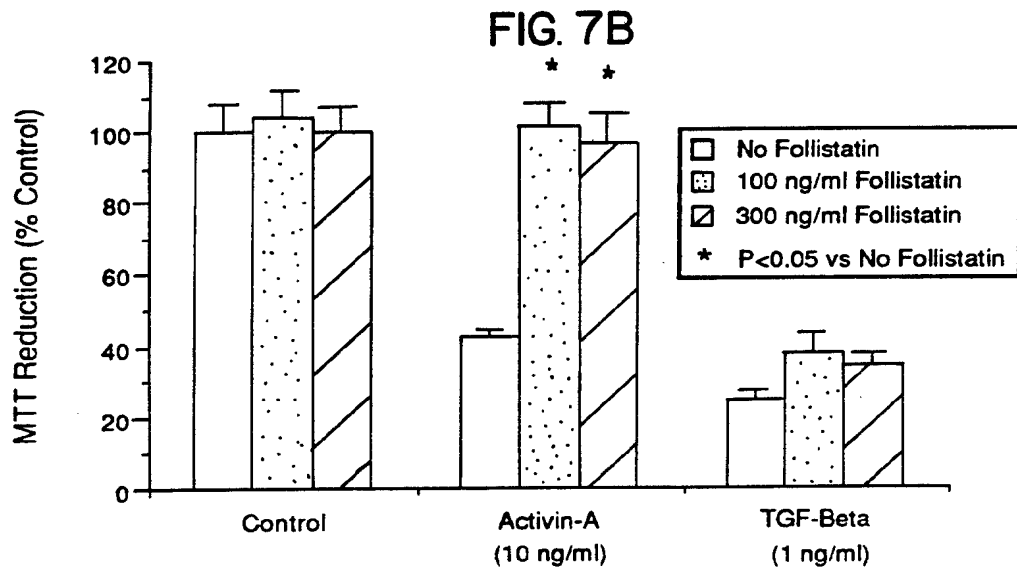
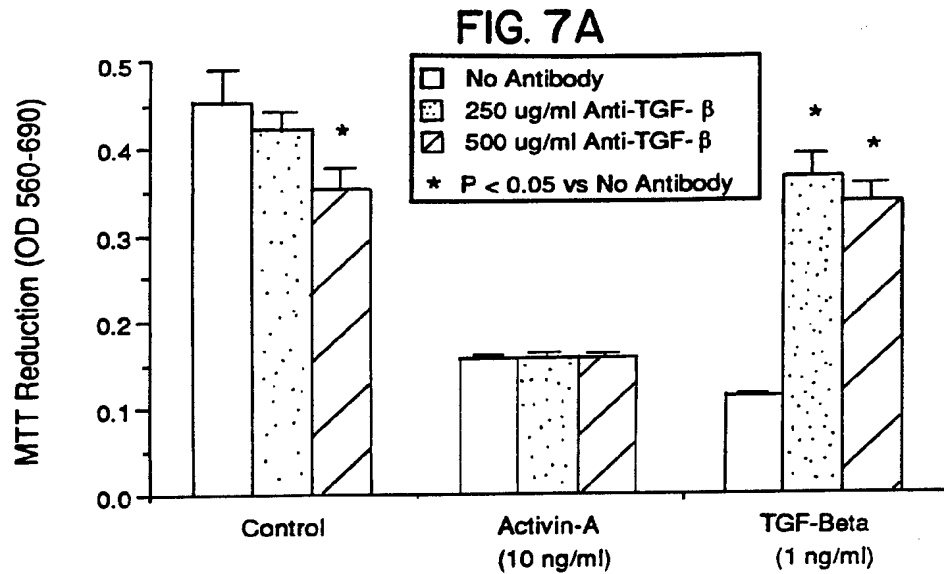


FIG. 8A

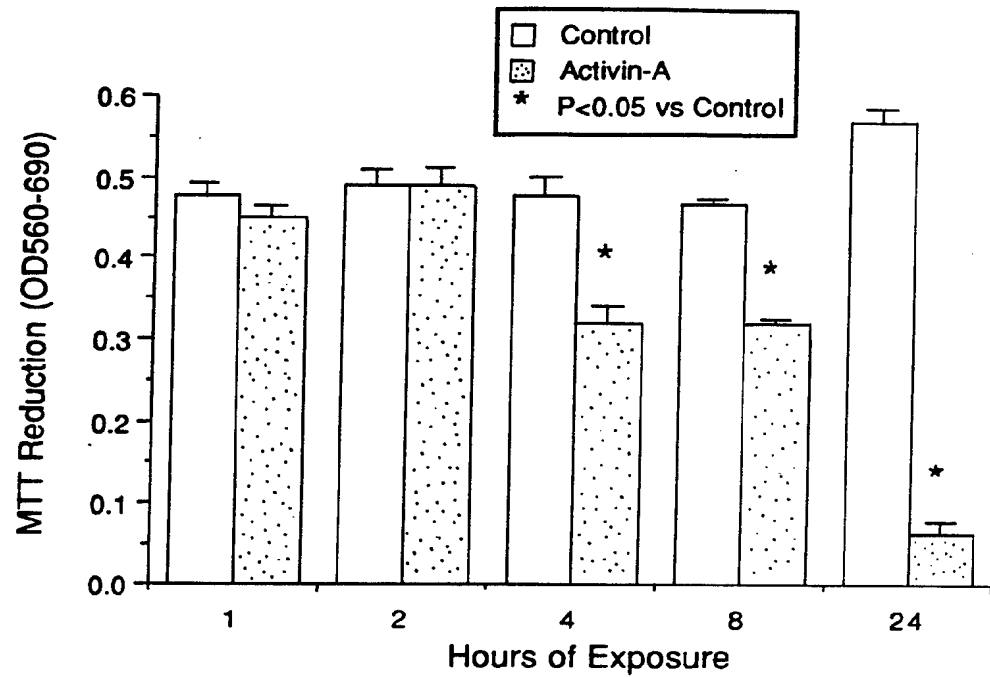
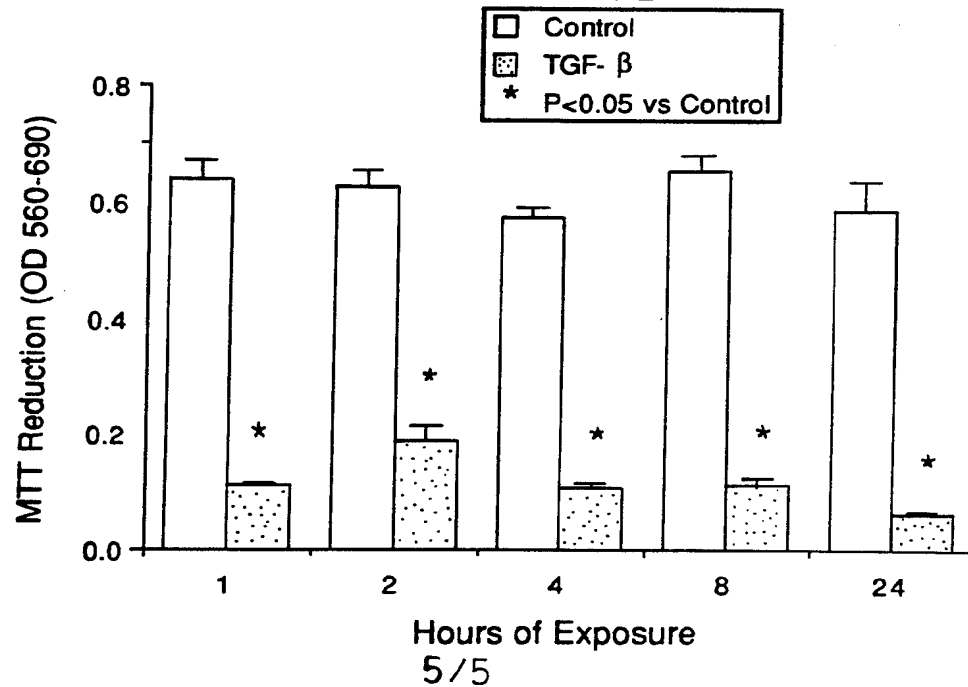


FIG. 8B



## INTERNATIONAL SEARCH REPORT

Interr. Application No

PCT/US 93/09885

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K37/02 A61K39/395 C07K15/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 08480 (CELTRIX LABORATORIES INC.) 29 May 1992 see page 29, line 9 - line 12 ---	1,9,10
P,X	HEPATOLOGY vol. 18, no. 2, August 1993 pages 347 - 356 R. SCHWALL ET AL. 'Activin induces cell death in hepatocytes in vivo and in vitro.' see the whole document ---	1,6,10
P,A	US,A,5 216 126 (COX ET AL.) 1 June 1993 see column 34, line 43 - column 36, line 9 -----	1,8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"Y" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

1 February 1994

Date of mailing of the international search report

21. 02. 94

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Authorized officer

Nooij, F

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/09885

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-11 are directed to a method of treatment of the animal/human body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 93/09885

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9208480	29-05-92	AU-A- 9046791	11-06-92
		CA-A- 2096441	17-05-92
		EP-A- 0557418	01-09-93
US-A-5216126	01-06-93	NONE	